Bioorganic & Medicinal Chemistry Letters, Vol.3, No.7, pp. 1409-1414, 1993
Printed in Great Britain

0960-894X/93 $6.00 + .00

THE STOICHIOMETRY OF BINDING OF FLAVIN MONONUCLEOTIDE (FMN) HYDROQUINONE TO *ESCHERICHIA COLI* CHORISMATE SYNTHASE

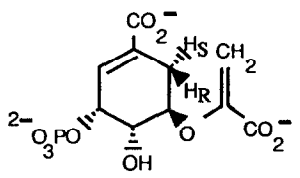
[a]Manoj K. Ramjee, [b]John R. Coggins, [c]Timothy R. Hawkes, [a]David J. Lowe and [a]Roger N.F. Thorneley*

[a]AFRC Institute of Plant Science Research, Nitrogen Fixation Laboratory, University of Sussex, Brighton BN1 9RQ, U.K.
[b]Dept. of Biochemistry, University of Glasgow, Glasgow G12 8QQ, U.K.
[c]ICI Agrochemicals, Jealotts Hill Research Station, Bracknell RG12 6EY, U.K.

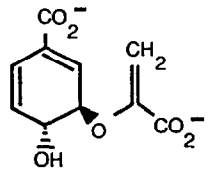
(*Received* 9 *October* 1992; *accepted* 16 *October* 1992)

Abstract: *Escherichia coli* chorismate synthase (EC 4.6.1.4), purified aerobically, does not contain oxidised flavin mononucleotide (FMN) as judged by its uv/visible and fluorescence spectra. However, transient kinetic studies of functioning enzyme show that each enzyme tetramer binds four equivalents of $FMNH_2$, the reduced hydroquinone state. The higher affinity of chorismate synthase for $FMNH_2$ (K_D<2 μM) relative to FMN (K_D>20 μM) is similar to that of bacterial luciferase.

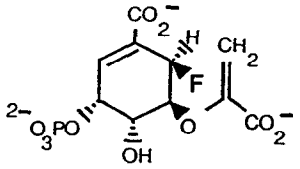
Chorismate synthase (5-enolpyruvylshikimate 3-phosphate phospholyase (EC 4.6.1.4)), the seventh enzyme in the shikimate pathway,[1] catalyses the conversion of 5-enolpyruvylshikimate 3-phosphate **1** (EPSP) to chorismate **2**, by the removal of the C-6 *pro*-R proton and elimination of phosphate.[2-5]



1



2



3

Although the conversion of **1** to **2** does not involve an overall oxidation or reduction, there is an essential requirement for a reduced flavin mononucleotide ($FMNH_2$) cofactor for enzyme activity.[6-9] Transient absorbance changes at 400 nm during single turnover experiments with **1** in the presence of sodium dithionite (required to reduce FMN to $FMNH_2$) have shown a direct involvement of $FMNH_2$ in the catalytic cycle.[10] The role of $FMNH_2$ in what is formally a *trans* 1-4 elimination reaction is intriguing and could involve novel free radical intermediates since a stable flavin semiquinone form of the enzyme has recently been detected using the substrate analogue *(6R)*-6 fluoro EPSP **3**.[11]

We now report the results of uv/visible absorbance and fluorescence emission spectroscopic experiments

to monitor oxidised FMN binding to apo-enzyme and a stopped-flow amplitude titration for functioning enzyme under reducing conditions with **1** as substrate that allows a stoichiometry for $FMNH_2$ binding to be determined.

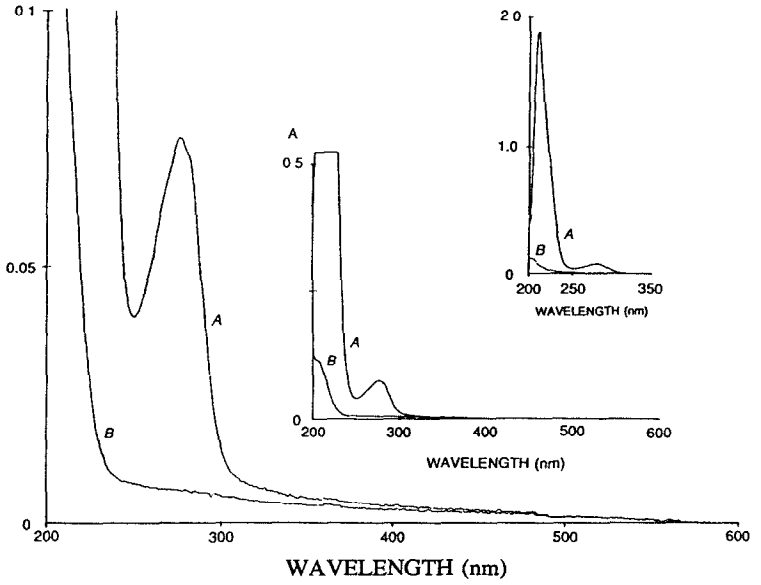


Figure 1. (*A*) UV/visible absorbance spectrum of *E. coli* chorismate synthase (97 μg ml^{-1}) in potassium phosphate buffer (50 mM, pH 7.5) containing 10% (v/v) glycerol. (*B*) As in *A* except enzyme omitted. Spectra recorded in 1 cm path length cuvette, 25°C in a Shimadzu MPS-2000 spectrophotometer. The inserts show the same spectra on condensed scales.

The uv/visible absorbance spectrum of *E. coli* chorismate synthase (Figure 1) suggests that no oxidised FMN is present in enzyme that has been purified aerobically. This was confirmed by electrospray mass spectrometry that gave a subunit M_r 39016.7 ± 4.5 which is very close to the value of M_r 39005.9 calculated from the amino acid composition derived from the DNA sequence data of Charles *et al.*[12] These data show that there are no covalently bound flavin or other prosthetic groups present in the enzyme.

Figure 2 shows that the near uv/visible spectrum of oxidised FMN (10 μM) does not change on addition of chorismate synthase (10 μM).[13] These data again suggest that oxidised FMN does not bind to the apo-enzyme under these conditions. Likewise the fluorescence emission spectra (460 to 600 nm with 365 nm excitation) of FMN at several concentrations in the range 0.2 to 10 μM were completely unaffected by the presence of 1 μM chorismate synthase. Figure 3 shows the maximum emission intensities (530 nm) of these spectra as a function of oxidised FMN concentration with coincident data points in the presence and absence of chorismate synthase. If oxidised FMN had bound to the apo-enzyme under these conditions the fluorescence of the FMN should have been at least partially quenched as has been reported for FMN binding to apo-flavodoxins.[14,15] We estimate a $K_D > 20$ μM for the binding of oxidised FMN to chorismate synthase.

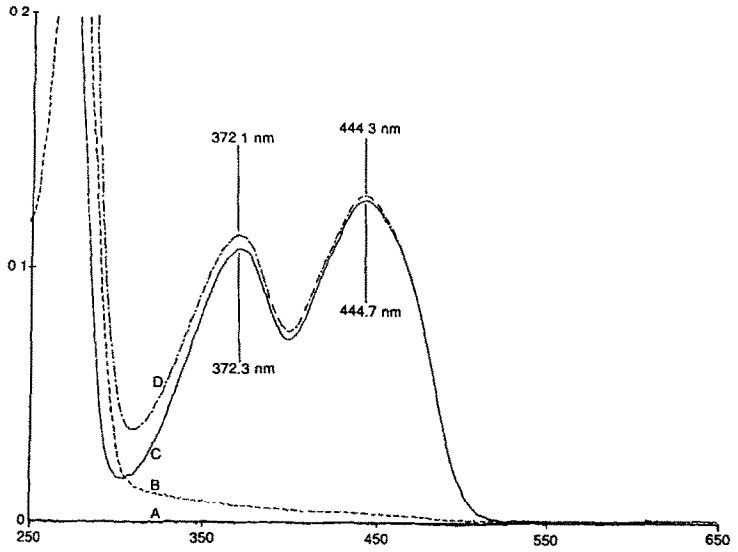


Figure 2. UV/visible spectra of FMN (10 μM) in the presence (**D**) and absence (**C**) of chorismate synthase (10 μM). The spectrum of the enzyme alone (**B**) accounts for the small differences in the two FMN spectra. All spectra were run in potassium phosphate buffer (50 mM, pH 7.0) in a 1 cm path length quartz cuvette. (**A**) spectrum of buffer.

Figure 4 shows the transient absorbance changes that occur at 400 nm when chorismate synthase, pre-incubated with four concentrations of $FMNH_2$ in the presence of dithionite ion (1 mM), was mixed in the stopped-flow spectrophotometer with EPSP (200 μM). A rapid increase in absorbance, essentially complete within the instrument dead time (3 ms) is followed by a quasi-steady-state phase before the absorbance decreases to its initial value. The area under each curve is essentially the same, but the amplitude and length of the steady state phase reflect the proportion of functioning enzyme with $FMNH_2$ bound. We have previously reported the difference spectrum relative to $FMNH_2$ of the intermediate giving rise to these absorbance changes.[10] The spectrum is shown as an insert in Figure 4. It resembles that of a flavin-C4a adduct, with a maximum at 400 nm, and is similar to that reported for mercuric ion reductase.[16] In the latter case, $FMNH_2$ reduces a disulphide bond in the enzyme to generate a thiolate that acts as a nucleophile to form the adduct at the C-4a position of the flavin. A similar mechanism can not occur with chorismate synthase since all four cysteine residues predicted by the Charles *et al.* sequence[12] have been detected as thiols.[10]

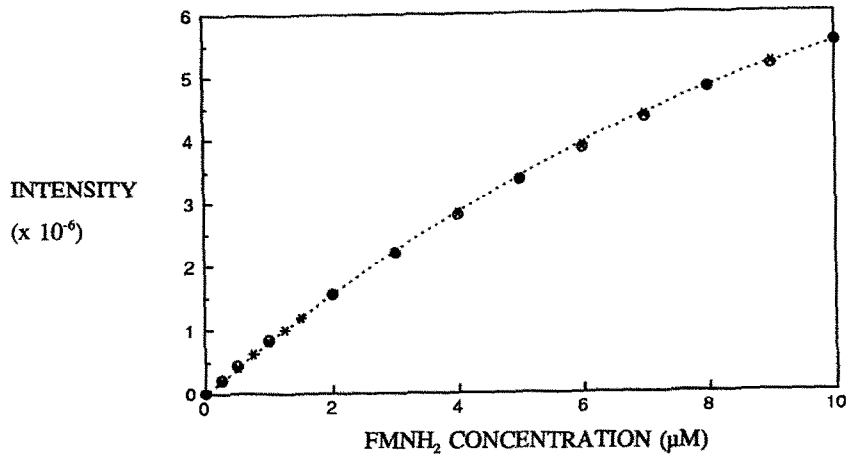


Figure 3. Fluorescence emission intensity (530 nm) of oxidised FMN in the presence (*) and absence (o) of chorismate synthase (1 μM) in potassium phosphate buffer (50 mM, pH 7.0).

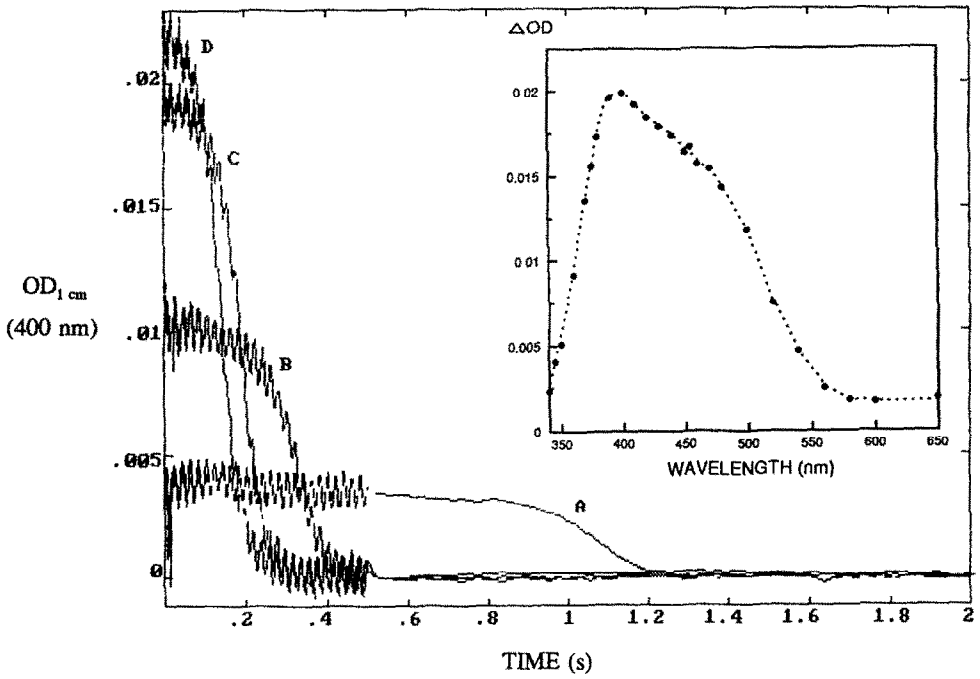


Figure 4. Stopped-flow absorbance changes (400 nm, 25°C) occurring when chorismate synthase (34 μM) pre-incubated with (A) 10 μM, (B) 20 μM, (C) 40 μM & (D) 80 μM FMNH$_2$ was mixed with EPSP (200 μM). Both syringes contained sodium dithionite (1 mM), potassium phosphate buffer (50 mM, pH 7.0). The insert shows the amplitude of traces similar to (D) as a function of wavelength.

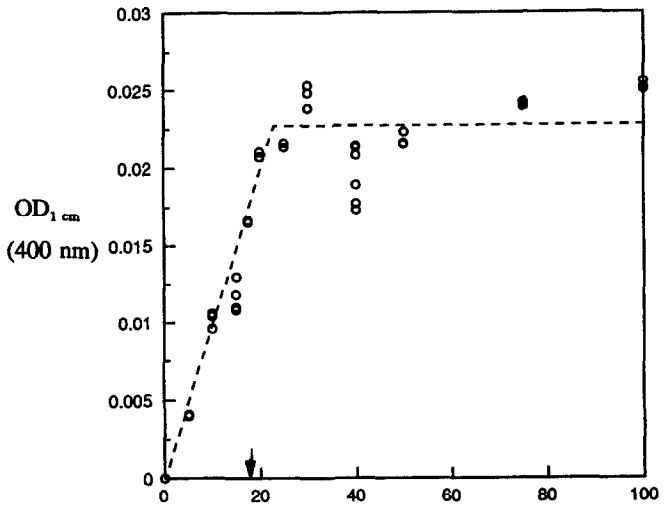


Figure 5. Stopped-flow amplitude data showing stoichiometric tight binding of $FMNH_2$ to each subunit of chorismate synthase.

The dependence of the amplitudes of traces such as those shown in Figure 4 on the final $FMNH_2$ concentration with a constant concentration of chorismate synthase (17 μM after mixing, indicated by the arrow) is shown in Figure 5. A linear increase in the amplitude occurs up to an $FMNH_2$ concentration of 20 μM, after which the amplitude remains constant. These data indicate that one equivalent of $FMNH_2$ binds to each subunit of the tetrameric enzyme. A $K_D < 2\mu M$ for $FMNH_2$ binding can be estimated from the linearity of the data in the $FMNH_2$ concentration range 5 to 20 μM.

The binding affinity of chorismate synthase for reduced $FMNH_2$ ($K_D < 2$ μM) is therefore at least ten times greater than its affinity for oxidised FMN ($K_D > 20$ μM). In this respect it resembles bacterial luciferase for which the relative affinities are *ca.* 100 fold.[17] It is also interesting to note that C(4a) hydroperoxy-dihydroflavin and C(4a) hydroxyflavin intermediates are proposed intermediates in the luciferase catalytic cycle[17,18] and that the only intermediate detected to date for chorismate synthase also resembles a C(4a)-flavin adduct.[10]

Acknowledgements: We thank Dr. C. Abell and Ms. A. Shneier for electrospray mass spectrometry at the Cambridge Centre for Molecular Recognition and the Science and Engineering Research Council (U.K.) for the award of a Case Studentship for M.R. in association with I.C.I. Agrochemicals, U.K.

References and Notes

1. Bentley, R. *Crit. Rev. Biochem. Mol. Biol.* **1990**, *25(5)*, 307-384.
2. Welch, G.R.; Cole, K.W.; Gaertner, F.H. *Arch. Biochem. Biophys.* **1974**, *165*, 505-518.
3. Hill, R.K.; Newkome, G.R. *J. Am. Chem. Soc.* **1969**, *91*, 5893-5894.
4. Onderka, D.K.; Floss, H.G. *J. Am. Chem. Soc.* **1969**, *91*, 5894-5896.
5. Floss, H.G.; Onderka, D.K.; Carroll, M. *J. Biol. Chem.* **1972**, *247(3)*, 736-744.
6. White, P.J.; Millar, G.; Coggins, J.R. *Biochem. J.* **1988**, *251*, 313-322.
7. Hasan, N.; Nester, E.W. *J. Biol. Chem.* **1978**, *253*, 4999-5004.
8. Schaller, A.; Windhofer, V.; Amrhein, N. *Arch. Biochem. Biophys.* **1990**, *282*, 437-442.
9. Mousdale, D.M.; Coggins, J.R. *FEBS. Lett.* **1986**, *205*, 328-332.
10. Ramjee, M.N.; Coggins, J.R.; Hawkes, T.R.; Lowe, D.J.; Thorneley, R.N.F. *J. Am. Chem. Soc.* **1991**, *113*, 8566-8567.
11. Ramjee, M.N.; Balasubramanian, S.; Abell, C.; Coggins, J.R.; Davies, G.M.; Hawkes, T.R., Lowe, D.J.; Thorneley, R.N.F. *J. Am. Chem. Soc.* **1992**, *114*, 3151-3153.
12. Charles, I.G.; Lamb, H.K.; Pickard, D.; Gordon, G.; Hawkins, A.R. *J. Gen. Microbiol.* **1990**, *136*, 353-358.
13. Chorismate synthase concentrations throughout this paper refer to the monomer (39kD) although the native enzyme is tetrameric (156 kD).
14. Mayhew, S.G.; Tollin, G. *Chemistry and Biochemistry of Flavodoxins, Part III*; Müller F.A., Ed; CRC Press: Boca Raton, Florida, U.S.A., 1992; pp. 389-426.
15. Fillat, M.F.; Edmonson, D.E.; Gomez-Moreno, C. *Biochim. Biophys. Acta.* **1990**, *1040*, 301-307.
16. Miller, S.M.; Massey, V.; Ballou, D.; Williams, C.H.; Distefano, M.D.; Moore, M.J.; Walsh, C.T. *Biochemistry.* **1990**, *29*, 2831-2841.
17. Baldwin, T.O.; Ziegler, M.M. *Chemistry and Biochemistry of Flavodoxins, Part III*; Müller F.A., Ed; CRC Press: Boca Raton, Florida, U.S.A., 1992; pp. 467-530.
18. Vervoort, J.; Müller, F.; Lee, J.; Van Den Berg, W.A.M.; Moonen, C.T.W. *Biochemistry.* **1986**, *25*, 8062-8070.